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1. Your reference — GWS\21755 01

2. Patent application number *(The Patent Office will fill in this part)* — 23 SEP 1999 — **9922558.3**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

   Microbiological Research Authority
   CAMR
   Porton Down
   Salisbury
   Wiltshire SP4 0JG

   Patents ADP number *(if you know it)*

   If the applicant is a corporate body, give the country/state of its incorporation — England

   6566160001

4. Title of the invention — Inhibition of secretion from non-neuronal cells

5. Name of your agent *(if you have one)*

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

   MATHYS & SQUIRE
   100 Grays Inn Road
   London WC1X 8AL

   1081001

   Patents ADP number *(if you know it)*

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

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   a) any applicant named in part 3 is not an inventor, or
   b) there is an inventor who is not named as an applicant, or
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Continuation sheets of this form	—
Description	22
Claim(s)	5
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Priority documents	—
Translations of priority documents	—
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	—
Request for preliminary examination and search *(Patents Form 9/77)*	—
Request for substantive examination *(Patents Form 10/77)*	—
Any other documents *(please specify)*	—

11. I/We request the grant of a patent on the basis of this application.

Signature: Matthy & Squire — Date: 23/9/99

12. Name and daytime telephone number of person to contact in the United Kingdom: G W Schlich — 0171 830 0000

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INHIBITION OF SECRETION FROM NON-NEURONAL CELLS

The present invention relates to treatment of disease by inhibition of cellular secretory processes, to agents and compositions therefor, and to manufacture of those agents and compositions. The present invention relates particularly, to treatment of diseases dependent upon the exocytotic activity of endocrine cells, exocrine cells, inflammatory cells, cells of the immune system, cells of the cardiovascular system and bone cells.

Exocytosis is the fusion of secretory vesicles with the plasma membrane and results in the discharge of vesicle content - a process also known as cell secretion. Exocytosis can be constitutive or regulated. Constitutive exocytosis is thought to occur in every cell type whereas regulated exocytosis occurs from specialised cells.

The understanding of the mechanisms involved in exocytosis has increased rapidly, following the proposal of the SNARE hypothesis (Rothman, 1994, Nature 372, 55-63). This hypothesis describes protein markers on vesicles, which recognise target membrane markers. These so-called cognate SNARES (denoted v-SNARE for vesicle and t-SNARE for target) facilitate docking and fusion of vesicles with the correct membranes, thus directing discharge of the vesicular contents into the appropriate compartment. Key to the understanding of this process has been the identification of the proteins involved. Three SNARE protein families have been identified for exocytosis: SNAP-25 and SNAP-23 and syntaxins are the t-SNARE families in the membrane and VAMPs (vesicle-associated membrane protein), including synaptobrevin and cellubrevin, the v-SNARE family on secretory vesicles. Key components of the fusion machinery including SNARES are involved in both regulated and constitutive exocytosis (De Camilli, 1993, Nature, 364, 387-388).

The clostridial neurotoxins are proteins with molecular masses of the order of 150kDa. They are produced by various species of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There are at present eight different classes of the neurotoxins known: tetanus toxin and botulinum neurotoxin in its serotypes A, B, C_1, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesized by the bacterium as a single polypeptide that is modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H) which has a molecular mass of approximately 100 kDa and the light chain (LC) which has a molecular mass of approximately 50 kDa. The clostridial neurotoxins are highly selective for neuronal cells and bind with high affinity.

The functional requirements of neurointoxication by the clostridial neurotoxins can be assigned to specific domains within the neurotoxin structure. The clostridial neurotoxins bind to an receptor site on the cell membrane of the motor neuron at the neuromuscular junction and, following binding to the highly specific receptor, are internalised by an endocytotic mechanism. The specific neuromuscular junction binding activity of clostridial neurotoxins is known to reside in the carboxy-terminal portion of the heavy chain component of the dichain neurotoxin molecule, a region known as H_C. The internalised clostridial neurotoxins possess a highly specific zinc-dependent endopeptidase activity that hydrolyses a specific peptide bond in at least one of three protein families, synaptobrevin, syntaxin or SNAP-25, which are crucial components of the neurosecretory machinery. The zinc-dependent endopeptidase activity of clostridial neurotoxins is found to reside in the L-chain (LC). The amino-terminal portion of the heavy chain component of the dichain neurotoxin molecule, a region known as H_N, is responsible for translocation of the neurotoxin, or a portion of it containing the endopeptidase activity, across the endosomal membrane following internalisation, thus allowing access of

the endopeptidase to the neuronal cytosol and its substrate protein(s). The result of neurointoxication is inhibition of neurotransmitter release from the target motor neuron due to prevention of release of synaptic vesicle contents.

The *mechanism by which the* H_N *domain effects translocation of the* endopeptidase into the neuronal cytosol is not fully characterised but is believed to involve a conformational change, insertion into the endosomal membrane and formation of some form of channel or pore through which the endopeptidase can gain access to the neuronal cytosol. Following binding to its specific receptor at the neuronal surface pharmacological and morphologic evidence indicate that the clostridial neurotoxins enter the cell by *endocytosis* (Black & Dolly, 1986, J. Cell Biol. 103, 535-44) *and then* have to pass through a low pH step for neuron intoxication to occur (Simpson et al, 1994, J. Pharmacol Exp. Ther., 269, 256-62). Acidic pH does not activate the toxin directly via a structural change, but is believed to trigger the process of LC membrane translocation from the neuronal endosomal vesicle lumen to the neuronal cytosol (Montecucco et al, 1994, FEBS Lett. 346, 92-98). There is a general consensus that toxin-*determined channels are related to the translocation process cytosol* (Schiavo & Montecucco, 1997, in Bacterial Toxins (ed. K. Aktories)). This model requires that the HC domain forms a transmembrane hydrophobic cleft across the acidic vesicle membrane that allows the partially unfolded LC passage through to the). The requisite conformational change is believed to be triggered by environmental factors in the neuronal endosomal compartment into which the neurotoxin is internalised.

Hormones are chemical messengers that are secreted by the endocrine glands of the body. They exercise specific physiological actions on other organs to which they are carried by the blood. The range of processes regulated by hormones includes various aspects of homeostasis (e.g. insulin regulates the concentration of glucose in the blood) growth (e.g.

growth hormone promotes growth and regulates fat, carbohydrate and protein metabolism) and maturation (e.g. sex hormones promote sexual maturation and reproduction). Endocrine hyperfunction results in disease conditions which are caused by excessive amounts of a hormone or hormones in the bloodstream. The causes of hyperfunction are classified as neoplastic, autoimmune, iatrogenic and inflammatory. The endocrine hyperfunction disorders are a complex group of diseases, not only because there is a large number of glands that can cause a pathology (e.g. anterior pituitary, posterior pituitary, thyroid, parathyroid, adrenal cortex, adrenal medulla, pancreas, ovaries, testis) but because many of the glands produce more than one hormone (e.g. the anterior pituitary produces corticotrophin, prolactin, luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone and gonadotrophins). The majority of disorders that cause hormone excess are due to neoplastic growth of hormone producing cells. However, certain tumours of non-endocrine origin can synthesise hormones causing endocrine hyperfunction disease symptoms. The hormone production under these conditions is termed "ectopic". Surgical removal or radiation induced destruction of part or all of the hypersecreting tissue is frequently the treatment of choice. However, these approaches are not always applicable, result in complete loss of hormone production or have to be repeated due to re-growth of the secreting tissue.

A further level of complexity in endocrine hyperfunction disorders arises in a group of conditions termed multiple endocrine neoplasia (MEN) where two or more endocrine glands are involved. The multiple endocrine neoplasia syndromes (MEN1 and MEN2) are familial conditions with an autosomal dominant pattern of inheritance. MEN1 is characterised by the association of parathyroid hyperplasia, pancreatic endocrine tumours, and pituitary adenomas, and has a prevalence of about 1 in 10000. MEN2 is the association of medullary cell carcinoma of the thyroid and phaeochromocytoma, though parathyroid hyperplasia may also occur in some sufferers.

Most of the morbidity associated with MEN1 is due to the effects of pancreatic endocrine tumours. Often surgery is not possible and the therapeutic aim is to reduce hormone excess. Aside from reducing tumour bulk, which is often precluded, inhibition of hormone secretion is the preferred course of action. Current procedures include subcutaneous application of the somatostatin analogue, octreotide. However, this approach is only temporarily effective, and the success diminishes over a period of months.

Many further disease states are known that involve secretion from other non-endocrine, non-neuronal cells. It would accordingly be desirable to treat, reduce or prevent secretion by non-neuronal cells, such as hyperfunction of the endocrine cells that causes or leads to these disease conditions.

The activity of the botulinum neurotoxins is to inhibit secretion, but is exclusively restricted to inhibition of neurotransmitter release from motor neurons.

It is known from WO 96/33273 that hybrid clostridial neurotoxin endopeptidases can be prepared and that these hybrids effectively inhibit release of a variety of neurotransmitters from other neuronal cells to which they are targeted, such as pain-transmitting neurones. These hybrids utilise the clostridial neurotoxin-derived translocation domain H_N, in addition to the endopeptidase light chain (LC).

Non-neuronal cells are, however, refractory to the effects of clostridial neurotoxins, since simple application of clostridial neurotoxins to the surface of non-neuronal cells does not lead to inhibition of secretory vesicle exocytosis. This insensitivity of non-neuronal cells to clostridial neurotoxins may be due to absence of the requisite receptor, absence of the correct internalisation & intracellular routing mechanism, or additional

factors that are not yet understood.

It is an object of the present invention to provide methods and compositions for inhibition of secretion from non-neuronal cells.

Accordingly, the present invention is based upon the use of a composition which inhibits the exocytotic machinery in neuronal cells and which surprisingly has been found to be effective at inhibiting exocytotic processes in non-neuronal cells.

A first aspect of the invention thus provides a method of inhibiting secretion from a non-neuronal cell comprising administering an agent comprising at least first and second domains, wherein the first domain cleaves one or more proteins essential to exocytosis and the second domain translocates the first domain into the cell.

Advantageously, the invention provides for inhibition of non-neuronal secretion and enables treatment of disease caused, exacerbated or maintained by such secretion.

An agent for use in the invention is suitably prepared by replacement of the cell-binding H_C domain of a clostridial neurotoxin with a ligand capable of binding to the surface of non-neuronal cells. Surprisingly, this agent is capable of inhibiting the exocytosis of a variety of secreted substances from non-neuronal cells. By covalently linking a clostridial neurotoxin, or a hybrid of two clostridial neurotoxins, in which the H_C region of the H-chain has been removed or modified, to a new molecule or moiety, the Targeting Moiety (TM), an agent is produced that binds to a binding site (BS) on the surface of the relevant non-neuronal secretory cells. A further surprising aspect of the present invention is that if the L-chain of a clostridial neurotoxin, or a fragment, variant or derivative of the L-chain containing the endopeptidase activity, is covalently linked to a TM which

can also effect internalisation of the L-chain, or a fragment of the endopeptidase activity, into the cytoplasm of a non-neuronal secretory cell, this also produces an agent capable of inhibiting secretion.

An example of an agent of the invention is a polypeptide comprising first and second domains, wherein said first domain cleaves one or more vesicle or plasma-membrane associated proteins essential to neuronal exocytosis and wherein said second domain translocates the polypeptide into the cell or translocates at least that portion responsible for the inhibition of exocytosis into the non-neuronal cell. The polypeptide can be derived from a neurotoxin in which case the polypeptide is typically free of clostridial neurotoxin and free of any clostridial neurotoxin precursor that can be converted into toxin by proteolytic action, being accordingly substantially non-toxic and suitable for therapeutic use. Accordingly, the invention may thus use polypeptides containing a domain equivalent to a clostridial toxin light chain and a domain providing the translocation function of the H_N of a clostridial toxin heavy chain, whilst lacking the functional aspects of a clostridial toxin H_C domain.

In use of the invention, the polypeptide is administered *in vivo* to a patient, the first domain is translocated into a non-neuronal cell by action of the second domain and cleaves one or more vesicle or plasma-membrane associated proteins essential to the specific cellular process of exocytosis, and cleavage of these proteins results in inhibition of exocytosis, thereby resulting in inhibition of secretion, typically in a non-cytotoxic manner.

The polypeptide of the invention may be obtained by expression of a recombinant nucleic acid, preferably a DNA, and can be a single polypeptide, that is to say not cleaved into separate light and heavy chain domains or two polypeptides linked for example by a disulphide bond.

The first domain preferably comprises a clostridial toxin light chain or a

functional fragment or variant of a clostridial toxin light chain. The fragment is optionally an N-terminal, or C-terminal fragment of the light chain, or is an internal fragment, so long as it substantially retains the ability to cleave the vesicle or plasma-membrane associated protein essential to exocytosis. The minimal domains necessary for the activity of the light chain of clostridial toxins are described in J. Biol. Chem., Vol.267, No. 21, July 1992, pages 14721-14729. The variant has a different peptide sequence from the light chain or from the fragment, though it too is capable of cleaving the vesicle or plasma-membrane associated protein. It is conveniently obtained by insertion, deletion and/or substitution of a light chain or fragment thereof. A variety of variants are possible, including (i) an N-terminal extension to a clostridial toxin light chain or fragment (ii) a clostridial toxin light chain or fragment modified by alteration of at least one amino acid (iii) a C-terminal extension to a clostridial toxin light chain or fragment, or (iv) combinations of 2 or more of (i)-(iii). In further embodiments of the invention, the variant contains an amino acid sequence modified so that (a) there is no protease sensitive region between the LC and H_N components of the polypeptide, or (b) the protease sensitive region is specific for a particular protease. This latter embodiment is of use if it is desired to activate the endopeptidase activity of the light chain in a particular environment or cell, though, in general, the polypeptides of the invention are in an active form prior to administration.

The first domain preferably exhibits endopeptidase activity specific for a substrate selected from one or more of SNAP-25, synaptobrevin/VAMP and syntaxin. The clostridial toxin from which this domain can be obtained or derived is preferably botulinum toxin or tetanus toxin. The polypeptide can further comprises a light chain or fragment or variant of one toxin type and a heavy chain or fragment or variant of another toxin type.

The second domain preferably comprises a clostridial toxin heavy chain H_N portion or a fragment or variant of a clostridial toxin heavy chain H_N

portion. The fragment is optionally an N-terminal or C-terminal or internal fragment, so long as it retains the function of the H_N domain. Teachings of regions within the H_N responsible for its function are provided for example in Biochemistry 1995, 34, pages 15175-15181 and Eur. J. Biochem, 1989, 185, pages 197-203. The variant has a different sequence from the H_N domain or fragment, though it too retains the function of the H_N domain. It is conveniently obtained by insertion, deletion and/or substitution of a H_N domain or fragment thereof, and examples of variants include (i) an N-terminal extension to a H_N domain or fragment, (ii) a C-terminal extension to a H_N domain or fragment, (iii) a modification to a H_N domain or fragment by alteration of at least one amino acid, or (iv) combinations of 2 or more of (i)-(iii). The clostridial toxin is preferably botulinum toxin or tetanus toxin.

In preparation of the polypeptides by recombinant means, methods employing fusion proteins can be employed, for example a fusion protein comprising a fusion of (a) a polypeptide of the invention as described above with (b) a second polypeptide adapted for binding to a chromatography matrix so as to enable purification of the fusion protein using said chromatography matrix. It is convenient for the second polypeptide to be adapted to bind to an affinity matrix, such as a glutathione Sepharose, enabling rapid separation and purification of the fusion protein from an impure source, such as a cell extract or supernatant.

One second purification polypeptide is glutathione-S-transferase (GST), and others may be chosen so as to enable purification on a chromatography column according to conventional techniques.

In a second aspect of the invention there is provided a method of inhibiting secretion from selected non-neuronal cells responsible for regulated secretion by administering an agent of the invention.

In a third aspect of the invention there is provided a method of treatment of disease resulting, or caused or maintained by secretions from non-neuronal cells, comprising administering an agent of the invention.

In further aspects of the invention there are provided agents of the invention targeted to non-neuronal cells responsible for secretion.

In one embodiment of the invention, an agent is provided for the treatment of conditions resulting from hyperfunction of endocrine cells, for example endocrine neoplasia.

Accordingly, an agent of the invention is used in the treatment of endocrine hyperfunction, to inhibit secretion of endocrine cell-derived chemical messengers. An advantage of the invention is that effective treatment of endocrine hyperfunction and associated disease states is now provided, offering relief to sufferers where hitherto there was none and no such agent available.

The agent preferably comprises a ligand or targeting domain which binds to an endocrine cell, and is thus rendered specific for these cell types. Examples of suitable ligands include iodine; thyroid stimulating hormone (TSH); TSH receptor antibodies; antibodies to the islet-specific monosialo-ganglioside GM2-1; insulin, insulin-like growth factor and antibodies to the receptors of both; TSH releasing hormone (protirelin) and antibodies to its receptor; FSH/LH releasing hormone (gonadorelin) and antibodies to its receptor; corticotrophin releasing hormone (CRH) and antibodies to its receptor; and ACTH and antibodies to its receptor. According to the invention, an endocrine targeted agent may thus be suitable for the treatment of a disease selected from: endocrine neoplasia including MEN; thyrotoxicosis and other diseases dependent on hypersecretions from the thyroid; acromegaly, hyperprolactinaemia, Cushings disease and other diseases dependent on anterior pituitary hypersecretion; hyperandrogenism,

chronic anovulation and other diseases associated with polycystic ovarian syndrome.

In a further embodiment, an agent of the invention is used for the treatment of conditions resulting from secretions of inflammatory cells, for example allergies. Ligands suitable to target agent to these cells include (i) for mast cells, complement receptors in general, including C4 domain of the Fc IgE, and antibodies/ligands to the C3a/C4a-R complement receptor; (ii) for eosinophils, antibodies/ligands to the C3a/C4a-R complement receptor, anti VLA-4 monoclonal antibody, anti-IL5 receptor, antigens or antibodies reactive toward CR4 complement receptor; (iii) for macrophages and monocytes, macrophage stimulating factor, (iv) for macrophages, monocytes and neutrophils, bacterial LPS and yeast B-glucans which bind to CR3, (v) for neutrophils, antibody to OX42, an antigen associated with the iC3b complement receptor, or IL8; (vi) for fibroblasts, mannose 6-phosphate/insulin-like growth factor-beta (M6P/IGF-II) receptor and PA2.26, antibody to a cell-surface receptor for active fibroblasts in mice. Diseases thus treatable according to the invention include diseases selected from allergies (seasonal allergic rhinitis (hay fever), allergic conjunctivitis, vasomotor rhinitis and food allergy), eosinophilia, asthma, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus erythematosus, ulcerative colitis, Crohn's disease, haemorrhoids, pruritus, glomerulonephritis, hepatitis, pancreatitis, gastritis, vasculitis, myocarditis, psoriasis, eczema, chronic radiation-induced fibrosis, lung scarring and other fibrotic disorders.

In a further embodiment, an agent of the invention is provided for the treatment of conditions resulting from secretions of the exocrine cells, for example acute pancreatitis (Hansen *et al*, 1999, J. Biol. Chem. 274, 22871-22876). Ligands suitable to target agent to these cells include pituitary adenyl cyclase activating peptide (PACAP-38) or an antibody to its receptor.

In a further embodiment, an agent of the invention is used for the treatment of conditions resulting from secretions of immunological cells, for example autoimmune disorders where B lymphocytes are to be targeted (immunosuppression). Ligands suitable to target agent to these cells include Epstein Barr virus fragment/surface feature or idiotypic antibody (binds to CR2 receptor on B-lymphocytes and lymph node follicular dendritic cells). Diseases treatable include myasthenia gravis, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus erythematosus, organ transplant, tissue transplant, fluid transplant, Graves disease, thyrotoxicosis, autoimmune diabetes, haemolytic anaemia, thrombocytopenic purpura, neutropenia, chronic autoimmune hepatitis, autoimmune gastritis, pernicious anaemia, Hashimoto's thyroiditis, Addison's disease, Sjogren's syndrome, primary biliary cirrhosis, polymyositis, scleroderma, systemic sclerosis, pemphigus vulgaris, bullous pemphigoid, myocarditis, rheumatic carditis, glomerulonephritis (Goodpasture type), uveitis, orchitis, ulcerative colitis, vasculitis, atrophic gastritis, pernicious anaemia, type1 diabetes mellitus.

In a further embodiment of the invention, an agent is provided for the treatment of conditions resulting from secretions of cells of the cardiovascular system. Suitable ligands for targeting platelets for the treatment of disease states involving inappropriate platelet activation and thrombus formation include thrombin and TRAP (thrombin receptor agonist peptide) or antibodies to CD31/PECAM-1, CD24 or CD106/VCAM-1, and ligands for targeting cardiovascular endothelial cells for the treatment of hypertension include GP1b surface antigen recognising antibodies.

In a further embodiment of the invention, an agent is provided for the treatment of bone disorders. Suitable ligands for targeting osteoblasts for the treatment of a disease selected from osteopetrosis and osteoporosis include calcitonin, and for targeting an agent to osteoclasts include osteoclast differentiation factor (TRANCE, or RANKL or OPGL) and antibody

to the receptor RANK.

A further specific embodiment of the present invention lies in treating mucus hypersecretion by administering a composition that inhibits mucus secretion by mucus secreting cells and/or inhibits neurotransmitter release from neuronal cells that control or direct mucus secretion. Specific disease states caused by or exacerbated by hypersecretion are localised to the airways, and are treatable by topical administration to the airways or to a selected region or to a selected portion of the airways of a compound that inhibits exocytosis in mucus secreting cells or in neurones that control or direct mucus secretion.

In use of the invention, a Targeting moiety (TM) provides specificity for the BS on the relevant non-neuronal secretory cells. The TM component of the agent can comprise one of many cell binding molecules, including, but not limited to, antibodies, monoclonal antibodies, antibody fragments (Fab, $F(ab)'_2$, Fv, ScFv, etc.), lectins, hormones, cytokines, growth factors, peptides, carbohydrates, lipids, glycons, nucleic acids or complement components.

It is known in the art that the H_C portion of the neurotoxin molecule can be removed from the other portion of the H-chain, known as H_N, such that the H_N fragment remains disulphide linked to the L-chain of the neurotoxin providing a fragment known as LH_N. Thus, in one embodiment of the present invention the LH_N fragment of a clostridial neurotoxin is covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H_C domain of a clostridial neurotoxin is mutated, blocked or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction. This modified clostridial

neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the heavy chain of a clostridial neurotoxin, in which the H_C domain is mutated, blocked or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction, is combined with the L-chain of a different clostridial neurotoxin. This hybrid, modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H_N domain of a clostridial neurotoxin is combined with the L-chain of a different clostridial neurotoxin. This hybrid LH_N is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is covalently linked, using linkages which may include one or more spacer regions, to a TM which can also effect the internalisation of the L-chain, or a fragment of the L-chain containing the endopeptidase activity, into the cytoplasm of the relevant endocrine cells responsible for secretion of chemical messengers.

In another embodiment of the invention, the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is covalently linked, using linkages which may include one or more spacer regions, to a translocation domain to effect transport of the endopeptidase fragment into the cytosol. Examples of translocation domains derived from bacterial neurotoxins are as follows:

Botulinum type A neurotoxin – amino acid residues (449-871)

Botulinum type B neurotoxin – amino acid residues (441-858)

Botulinum type C neurotoxin	– amino acid residues (442-866)
Botulinum type D neurotoxin	– amino acid residues (446-862)
Botulinum type E neurotoxin	– amino acid residues (423-845)
Botulinum type F neurotoxin	– amino acid residues (440-864)
Botulinum type G neurotoxin	– amino acid residues (442-863)
Tetanus neurotoxin	– amino acid residues (458-879)

A further option is for the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, to be expressed recombinantly as a fusion protein with a TM which can also effect the internalisation of the L-chain, or a fragment of the L-chain containing the endopeptidase activity, into the cytoplasm of the relevant endocrine cells responsible for secretion of chemical messengers. The expressed fusion protein may also include one or more spacer regions. Nucleic acid encoding the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, may also associated with a TM which can also effect the internalisation of the nucleic acid encoding the L-chain, or a fragment of the L-chain containing the endopeptidase activity, into the cytoplasm of the relevant non-neuronal cells responsible for secretion of chemical messengers. Ideally, the coding sequence should be expressed.

The invention further provides pharmaceutical compositions comprising an agent or a conjugate of the invention and a pharmaceutically acceptable carrier.

In therapeutic use the agent or conjugate will normally be employed in the form of a pharmaceutical composition in association with a human pharmaceutical carrier, diluent and/or excipient, although the exact form of the composition will depend on the mode of administration.

The dosage ranges for administration of the compounds of the present

invention are those to produce the desired therapeutic effect. It will be appreciated that the dosage range required depends on the precise nature of the conjugate, the route of administration, the nature of the formulation, the age of the patient, the nature, extent or severity of the patient's condition, contraindications, if any, and the judgement of the attending physician. Wide variations in the required dosage, however, are to be expected depending on the precise nature of the conjugate. Variations in these dosage levels can be adjusted using standard empirical routines for optimisation, as is well understood in the art.

Fluid unit dosage forms are typically prepared utilising a pyrogen-free sterile vehicle. The active ingredient, depending on the vehicle and concentration used, can be either dissolved or suspended in the vehicle, the solution being made isotonic if necessary by addition of sodium chloride and sterilised by filtration through a sterile filter using aseptic techniques before filling into suitable sterile vials or ampoules and sealing. Alternatively, if solution stability is adequate, the solution in its sealed containers may be sterilised by autoclaving. Advantageously additives such as buffering, solubilising, stabilising, preservative or bactericidal, suspending or emulsifying agents and/or local anaesthetic agents may be dissolved in the vehicle.

Dry powders which are dissolved or suspended in a suitable vehicle prior to use may be prepared by filling pre-sterilised drug substance and other ingredients into a sterile container using aseptic technique in a sterile area. Alternatively the ingredients may be dissolved into suitable containers using aseptic technique in a sterile area. The product is then freeze dried and the containers are sealed aseptically.

The agent described in this invention can be used *in vivo*, either directly or as a pharmaceutically acceptable salt, for the treatment of conditions involving secretion from non-neuronal cells, such as hypersecretion of

endocrine cell derived chemical messengers, hypersecretion from exocrine cells, secretions from the cells of the immune system, the cardiovascular system and from bone cells.

The present invention will now be described by reference to the following examples illustrated by the accompanying drawings in which:-

Fig. 1 shows SDS-PAGE analysis of WGA-LH_N/A purification scheme;

Fig. 2 shows activity of WGA-LH_N/A on release of transmitter from HIT-T15 cells;

Fig. 3 shows correlation of SNAP-25 cleavage with inhibition of neurotransmitter release following application of WGA-LH_N/A to HIT-T15 cells; and

Fig. 4 shows activity of WGA-LH_N/A on release of [^{3}H]-noradrenaline from undifferentiated PC12 cells

Example 1.

Production of a conjugate of a lectin from *Triticum vulgaris* and LH_N/A

Materials

Lectin from *Triticum vulgaris* (WGA) was obtained from Sigma Ltd.

LH_N/A was prepared essentially by the method of Shone C.C., Hambleton, P., and Melling, J. 1987, *Eur. J. Biochem.* 167, 175-180.

SPDP was from Pierce Chemical Co.

PD-10 desalting columns were from Pharmacia.

Dimethylsulphoxide (DMSO) was kept anhydrous by storage over a molecular sieve.

Denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and non-denaturing polyacrylamide gel electrophoresis was performed using gels and reagents from Novex.

Additional reagents were obtained from Sigma Ltd

Methods

The lyophilised lectin was rehydrated in phosphate buffered saline (PBS) to a final concentration of 10 mg/ml. Aliquots of this solution were stored at -20°C until use.

The WGA was reacted with an equal concentration of SPDP by the addition of a 10 mM stock solution of SPDP in DMSO with mixing. After one hour at room temperature the reaction was terminated by desalting into PBS over a PD-10 column.

The thiopyridone leaving group was removed from the product to release a free -SH group by reduction with dithiothreitol (DTT; 5 mM; 30 min). The thiopyridone and DTT were removed by once again desalting into PBS over a PD-10 column.

The LH_N/A was desalted into PBSE (PBS containing 1 mM EDTA). The resulting solution (0.5-1.0 mg/ml) was reacted with a four-fold molar excess of SPDP by addition of a 10 mM stock solution of SPDP in DMSO. After 3 h at room temperature the reaction was terminated by desalting over a PD-10 column into PBSE.

A portion of the derivatized LH_N/A was removed from the solution and reduced with DTT (5 mM, 30 min). This sample was analyzed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatisation. The degree of derivatisation achieved was 3.53 ± 0.59 mol/mol.

The bulk of the derivatized LH_N/A and the derivatized WGA were mixed in proportions such that the ExL was in greater than three-fold molar excess. The conjugation reaction was allowed to proceed for >16 h at 4°C.

The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by centrifugation through concentrators (with 10000 molecular weight exclusion limit) before application to a Superose 12 column on an FPLC chromatography system (Pharmacia). The column was eluted with PBS and the elution profile followed at 280 nm.

Fractions were analyzed by SDS-PAGE on 4-20% polyacrylamide gradient gels, followed by staining with Coomassie Blue. The major conjugate products have an apparent molecular mass of between 106-150 kDa, these are separated from the bulk of the remaining unconjugated LH_N/A and more completely from the unconjugated WGA. Fractions containing conjugate were pooled prior to addition to PBS-washed N-acetylglucosamine-agarose. Lectin-containing proteins (i.e. WGA-LH_N/A conjugate) remained bound to the agarose during washing with PBS to remove contaminants (predominantly unconjugated LH_N/A). WGA-LH_N/A conjugate was eluted from the column by the addition of 0.3M N-acetylglucosamine (in PBS) and the elution profile followed at 280 nm. See Fig 1 for SDS-PAGE profile of the whole purification scheme.

The fractions containing conjugate were pooled, dialysed against PBS, and stored at 4°C until use.

Example 2

Activity of WGA-LH_N/A in cultured endocrine cells (HIT-T15)

The hamster pancreatic B cell line HIT-T15 is an example of a cell line of endocrine origin. It thus represents a model cell line for the investigation of inhibition of release effects of the agents. HIT-T15 cells possess surface moieties that allow for the binding, and internalisation, of WGA-LH_N/A. Figure 2 illustrates the inhibition of release of insulin from HIT-T15 cells after prior incubation with WGA-LH_N/A. It is clear that dose-dependent inhibition is observed, indicating that WGA-LH_N/A can inhibit the release of insulin from an endocrine cell model.

Inhibition of insulin release was demonstrated to correlate with cleavage of the SNARE protein, SNAP-25 (Figure 3). Thus, inhibition of release of chemical messenger is due to a clostridial endopeptidase-mediated effects of SNARE-protein cleavage.

Materials

Insulin radioimmunoassay kits were obtained from Linco Research Inc., USA.

Western blotting reagents were obtained from Novex.

Methods

HIT-T15 cells were seeded onto 12 well plates and cultured in RPMI-1640 medium containing 5% foetal bovine serum, 2mM L-glutamine for 5 days prior to use. WGA-LH_N/A was applied for 4 hours on ice, the cells were washed to remove unbound WGA-LH_N/A, and the release of insulin assayed 16 hours later. The release of insulin from HIT-T15 cells was assessed by

radioimmunoassay exactly as indicated by the manufacturers' instructions.

Cells were lysed in 2M acetic acid / 0.1% TFA. Lysates were dried then resuspended in 0.1M Hepes, pH 7.0. To extract the membrane proteins Triton-X-114 (10%, v/v) was added and incubated at 4°C for 60 min. The insoluble material was removed by centrifugation and the supernatants were warmed to 37°C for 30 min. The resulting two phases were separated by centrifugation and the upper phase discarded. The proteins in the lower phase were precipitated with chloroform/methanol for analysis by Western blotting.

The samples were separated by SDS-PAGE and transferred to nitrocellulose. Proteolysis of SNAP-25, a crucial component of the neurosecretory process and the substrate for the zinc-dependent endopeptidase activity of BoNT/A, was then detected by probing with an antibody (SMI-81) that recognises both the intact and cleaved forms of SNAP-25.

Example 3

Activity of WGA-LH_N/A in cultured neuroendocrine cells (PC12)

The rat pheochromocytoma PC12 cell line is an example of a cell line of neuroendocrine origin. In its undifferentiated form it has properties associated with the adrenal chromaffin cell (Greene and Tischler, in "Advances in Cellular Neurobiology".(Federoff and Hertz, eds), Vol. 3, p373-414. Academic Press, New York, 1982). It thus represents a model cell line for the investigation of inhibition of release effects of the agents. PC12 cells possess surface moieties that allow for the binding, and internalisation, of WGA-LH_N/A. Figure 4 illustrates the inhibition of release of insulin from PC12 cells after prior incubation with WGA-LH_N/A. It is clear that dose-dependent inhibition is observed, indicating that WGA-LH_N/A can inhibit the release of transmitter from a neuroendocrine cell

model. Comparison of the inhibition effects observed with conjugate and the untargeted LH_N/A demonstrate the requirement for a targeting moiety (TM) for efficient inhibition of transmitter release.

Methods

PC12 cells were cultured on 24 well plates in RPMI-1640 medium containing 10% horse serum, 5% foetal bovine serum, 1% L-glutamine. Cells were treated with a range of concentrations of WGA-LH_N/A for three days. Secretion of noradrenaline was measured by labelling cells with [^{3}\H]-noradrenaline (2μCi/ml, 0.5ml/well) for 60 min. Cells were washed every 15 min for 1 hour then basal release determined by incubation with a balanced salt solution containing 5mM KCl for 5 min. Secretion was stimulated by elevating the concentration of extracellular potassium (100mM KCl) for 5 min. Radioactivity in basal and stimulated superfusates was determined by scintillation counting. Secretion was expressed as a percentage of the total uptake and stimulated secretion was calculated by subtracting basal. Inhibition of secretion was dose-dependent with an observed IC_{50} of $0.63 \pm 0.15\mu g/ml$ ($n = 3$). Inhibition was significantly more potent when compared to untargeted endopeptidase (LH_N/A in Fig. 4). Thus WGA-LH_N/A inhibits release of neurotransmitter from a model neuroendocrine cell type.

CLAIMS

1. A method of inhibiting secretion from a non-neuronal cell comprising administering an agent comprising at least first and second domains, wherein the first domain cleaves one or more proteins essential to exocytosis and the second domain translocates the first domain into the cell.

2. A method according to Claim 1, for treatment of disease caused, exacerbated or maintained by secretion from a non-neuronal cell or non-neuronal cells.

3. A method according to Claim 1 or 2, wherein the agent further comprises a third domain for targeting the agent to a non-neuronal cell.

4. A method according to Claim 3 wherein the third domain targets the agent to an endocrine cell.

5. A method according to Claim 4 wherein the third domain comprises or consists of a ligand selected from iodine; thyroid stimulating hormone (TSH); TSH receptor antibodies; antibodies to the islet-specific monosialo-ganglioside GM2-1; insulin, insulin-like growth factor and antibodies to the receptors of both; TSH releasing hormone (protirelin) and antibodies to its receptor; FSH/LH releasing hormone (gonadorelin) and antibodies to its receptor; corticotrophin releasing hormone (CRH) and antibodies to its receptor; and ACTH and antibodies to its receptor.

6. A method according to Claim 4 or 5 for treatment of a disease selected from endocrine neoplasia including MEN; thyrotoxicosis and other diseases dependent on hypersecretions from the thyroid; acromegaly, hyperprolactinaemia, Cushings disease and other diseases dependent on anterior pituitary hypersecretion; hyperandrogenism, chronic anovulation

and other diseases associated with polycystic ovarian syndrome.

7. A method according to Claim 3 wherein the third domain targets the agent to inflammatory cells

8. A method according to Claim 7 wherein the third domain comprises or consists of a ligand selected from (i) for mast cells, complement receptors in general, including C4 domain of the Fc IgE, and antibodies/ligands to the C3a/C4a-R complement receptor; (ii) for eosinophils, antibodies/ligands to the C3a/C4a-R complement receptor, anti VLA-4 monoclonal antibody, anti-IL5 receptor, antigens or antibodies reactive toward CR4 complement receptor; (iii) for macrophages and monocytes, macrophage stimulating factor, (iv) for macrophages, monocytes and neutrophils, bacterial LPS and yeast B-glucans which bind to CR3, (v) for neutrophils, antibody to OX42, an antigen associated with the iC3b complement receptor, or IL8; (vi) for fibroblasts, mannose 6-phosphate/insulin-like growth factor-beta (M6P/IGF-II) receptor and PA2.26, antibody to a cell-surface receptor for active fibroblasts in mice.

9. A method according to Claim 7 or 8 for treatment of a disease selected from allergies (seasonal allergic rhinitis (hay fever), allergic conjunctivitis, vasomotor rhinitis and food allergy), eosinophilia, asthma, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus erythematosus, ulcerative colitis, Crohn's disease, haemorrhoids, pruritus, glomerulonephritis, hepatitis, pancreatitis, gastritis, vasculitis, myocarditis, psoriasis, eczema, chronic radiation-induced fibrosis, lung scarring and other fibrotic disorders.

10. A method according to Claim 3 wherein the third domain targets the agent to an exocrine cell.

11. A method according to Claim 10 wherein the third domain comprises

or consists of a ligand selected from pituitary adenyl cyclase activating peptide (PACAP-38) and an antibody to its receptor.

12. A method according to Claim 10 or 11 for treatment of acute pancreatitis.

13. A method according to Claim 3 wherein the third domain targets the agent to immunological cells.

14. A method according to Claim 13 wherein the third domain comprises or consists of a ligand selected from Epstein Barr virus fragment/surface feature and idiotypic antibody (binds to CR2 receptor on B-lymphocytes and lymph node follicular dendritic cells).

15. A method according to Claim 13 or 14 for treatment of a disease selected from myasthenia gravis, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus erythematosus, organ transplant, tissue transplant, fluid transplant, Graves disease, thyrotoxicosis, autoimmune diabetes, haemolytic anaemia, thrombocytopenic purpura, neutropenia, chronic autoimmune hepatitis, autoimmune gastritis, pernicious anaemia, Hashimoto's thyroiditis, Addison's disease, Sjogren's syndrome, primary biliary cirrhosis, polymyositis, scleroderma, systemic sclerosis, pemphigus vulgaris, bullous pemphigoid, myocarditis, rheumatic carditis, glomerulonephritis (Goodpasture type), uveitis, orchitis, ulcerative colitis, vasculitis, atrophic gastritis, pernicious anaemia, and type1 diabetes mellitus.

16. A method according to Claim 3 wherein the third domain targets the agent to cells of the cardiovascular system.

17. A method according to Claim 16 wherein the third domain comprises or consists of a ligand selected, for targeting platelets for the treatment of

disease states involving inappropriate platelet activation and thrombus formation, from thrombin and TRAP (thrombin receptor agonist peptide) and antibodies to CD31/PECAM-1, CD24 or CD106/VCAM-1, and for targeting cardiovascular endothelial cells for the treatment of hypertension, from GP1b surface antigen recognising antibodies.

18. A method according to Claim 3 wherein the third domain targets the agent to a cell whose secretions can lead to bone disorders.

19. A method according to Claim 18 wherein the third domain comprises or consists of a ligand selected, for targeting osteoblasts for the treatment of a disease selected from osteopetrosis and osteoporosis, from calcitonin, and for targeting an agent to osteoclasts, from osteoclast differentiation factor (TRANCE, or RANKL or OPGL) and antibody to the receptor RANK.

20. A method according to any previous Claim, wherein the agent comprises a first domain that cleaves a protein selected from SNAP-25, synaptobrevin and syntaxin.

21. A method according to Claim 20 wherein the first domain comprises a light chain of a clostridial neurotoxin, or a fragment, variant or derivative thereof which inhibits exocytosis.

22. A method according to any previous Claim, wherein the second domain comprises a H_N region of a botulinum polypeptide, or a fragment, variant or derivative thereof that translocates the exocytosis inhibiting activity of the first domain into the cell.

23. A method according to any previous Claim for inhibition of constitutive and regulated release from non-neuronal cells.

24. An agent for inhibiting secretion from a non-neuronal cell, comprising

at least first, second and third domains, wherein the first domain cleaves one or more proteins essential to exocytosis, the second domain translocates the first domain into the cell and the third domain binds to a non-neuronal cell.

25. An agent according to Claim 24, wherein the third domain is as defined in any of Claims 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 18 and 19.

26. A pharmaceutical composition comprising an agent according to Claim 24 or 25 in combination with a pharmaceutically acceptable carrier.

27. Use of an agent according to Claim 24 or 25 in treatment of a disease caused, exacerbated or maintained by secretion from a non-neuronal cell.

28. Use of an agent according to Claim 24 or 25 in manufacture of a medicament for treatment of a disease caused, exacerbated or maintained by secretion from a non-neuronal cell.

Figure 1

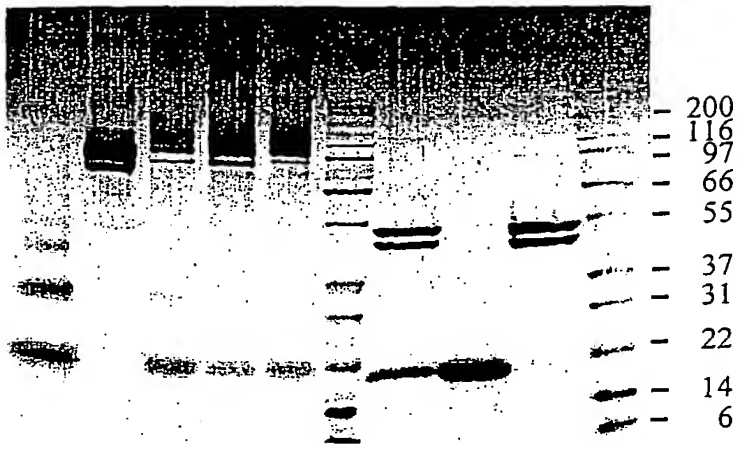

FIG. 1 SDS-PAGE analysis of WGA-LH_N/A purification scheme. Protein fractions were subjected to 4-20% polyacrylamide SDS-PAGE prior to staining with Coomassie blue. Lanes 6-8 were run in the presence of 0.1M DTT. Lanes 1 (&7) and 2 (& 8) represent derivatised WGA and derivatised LH_N/A respectively. Lanes 3-5 represent conjugation mixture, post-Superose-12 chromatography and post GlcNAc-affinity chromatography respectively. Lanes 6 represents a sample of reduced final material. Approximate molecular masses (kDa) are indicated on the Figure.

Figure 2

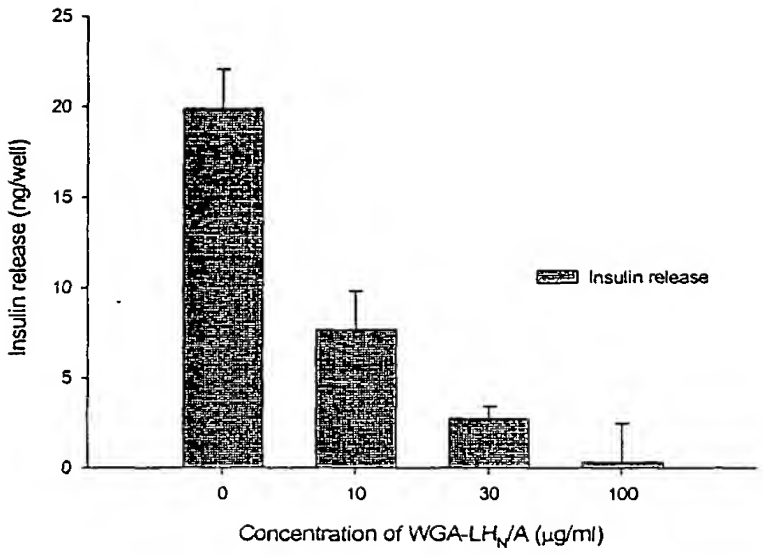

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FIG. 2. Activity of WGA-LH$_N$/A on release of transmitter from HIT-T15 cells. Cells were exposed to varying concentrations of WGA-LH$_N$/A on-ice for four hours. The cells were washed and incubated for 16 hours at 37°C prior to the determination of insulin release. Each point shown is the mean of at least three determinations + SE (release)

Figure 3

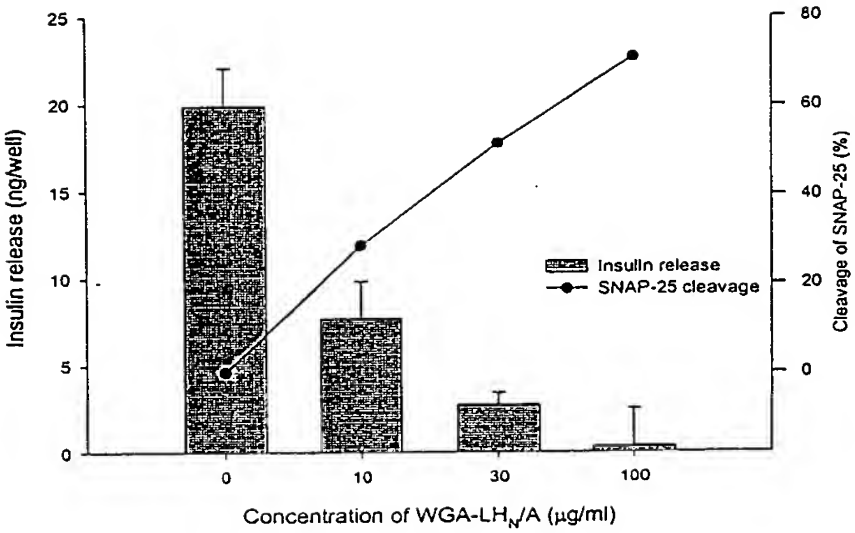

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FIG. 3. WGA-LH$_N$/A dependent cleaveage of SNAP-25 in HIT-T15 cells correlates with inhibition of insulin release. Cells were exposed to varying concentrations of WGA-LH$_N$/A on-ice for four hours. The cells were washed and incubated for 16 hours at 37°C prior to the determination of insulin release and SNAP-25 cleavage. Each point shown is the mean of at least three determinations + SE (release) or two determinations (cleavage).

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Figure 4

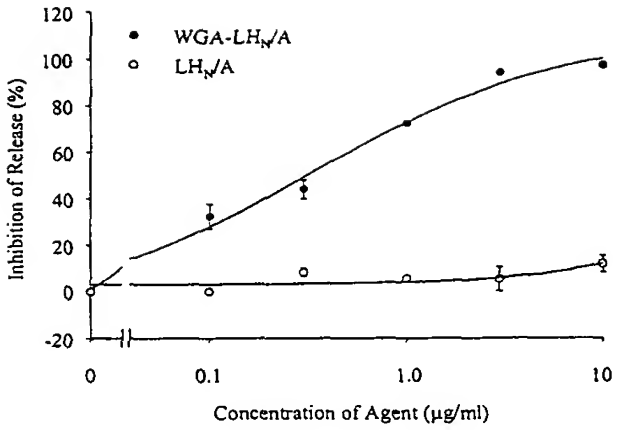

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FIG. 4. Activity of WGA-LH$_N$/A on release of [^{3}H]-noradrenaline from undifferentiated PC12 cells. Cells were exposed to varying concentrations of WGA-LH$_N$/A for three days prior to determination of [^{3}H]-noradrenaline release. Each point shown is the mean of at least three determinations + SE.

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